Plant Proteins Involved in *Agrobacterium*-Mediated Genetic Transformation

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plant genetic transformation, virulence effector proteins, T-DNA, host response to transformation

Abstract

Agrobacterium species genetically transform plants by transferring a region of plasmid DNA, T-DNA, into host plant cells. The bacteria also transfer several virulence effector proteins. T-DNA and virulence proteins presumably form T-complexes within the plant cell. Super-T-complexes likely also form by interaction of plant-encoded proteins with T-complexes. These protein-nucleic acid complexes traffic through the plant cytoplasm, enter the nucleus, and eventually deliver T-DNA to plant chromatin. Integration of T-DNA into the plant genome establishes a permanent transformation event, permitting stable expression of T-DNA-encoded transgenes. The transformation process is complex and requires participation of numerous plant proteins. This review discusses our current knowledge of plant proteins that contribute to *Agrobacterium*-mediated transformation, the roles these proteins play in the transformation process, and the modern technologies that have been employed to elucidate the cell biology of transformation.

INTRODUCTION

For more than a century since their discovery as agents of disease (97), *Agrobacterium* species have fascinated microbiologists, plant pathologists, molecular geneticists, and (most recently) agricultural biotechnologists. Virulent strains of *Agrobacterium* cause a variety of neoplasms, including crown gall disease (*A. tumefaciens* and *A. vitis*), hairy root disease (*A. rhizogenes*), and cane gall disease (*A. rubi*), on numerous plant species. More recently, disarmed (nontumorigenic) *Agrobacterium* strains have provided a means to produce genetically modified plants.

The molecular mechanism of plant transformation, resulting in either tumor formation or the generation of transgenic plants, initiates with the processing of a region, the transferred DNA (T-DNA), of a large resident *Agrobacterium* plasmid [the tumor inducing (Ti) or root inducing (Ri) plasmid], and the transfer of T-DNA to plant cells. Although several *Agrobacterium* chromosomal genes participate in these initial events, generation of single-strand T-DNA molecules (T-strands) and T-strand transport to plant cells occurs predominantly as a result of the activity of *Agrobacterium* virulence (Vir) proteins. VirD1 and VirD2 nick the Ti/Ri-plasmid at T-DNA border repeat sequences, following which VirD2 covalently links to the 5′ end of the resulting T-strand. VirD2/T-strands exit the bacterium through a type IV protein secretion system (T4SS) made up of 11 VirB proteins and VirD4 (41). For recent reviews on T-DNA processing and transfer see 18, 23, 46, 73.

VirD2 is not the only Vir protein transferred to plants. *Agrobacterium* also transfers VirD5, VirE2, VirE3, VirF, and (in the case of some *A. rhizogenes* strains) GALLS-FL and GALLS-CT (53, 86, 94, 98, 117, 118, 119). These effector proteins likely form complexes with VirD2/T-strands in the plant cell and also interact with several plant proteins. These Vir protein interacting partners, along with other host proteins, are important for successful transformation. The identification of these host proteins, along with characterization of the roles they play in *Agrobacterium*-mediated plant genetic transformation, are the subject of this review.

Transferred DNA (T-DNA): when within the Ti-plasmid, it is known as the T-DNA region

T-strand: single-stranded DNA that is processed from the T-DNA region of the Ti-plasmid and is transferred to the host cell

Ti/Ri-plasmid: tumor/root-inducing plasmid that is maintained in all virulent *Agrobacterium tumefaciens* and *A. rhizogenes* strains

T4SS: type IV secretion system

Vir protein: virulence protein. The genes encoding Vir proteins are located on the Ti-plasmid, but these genes are not normally transferred to the host

***Agrobacterium* effector protein:** protein transferred from *Agrobacterium* to host cells that mediates transformation

APPROACHES TO IDENTIFYING PLANT GENES INVOLVED IN *AGROBACTERIUM*-MEDIATED TRANSFORMATION

Plants differ greatly in their susceptibility to *Agrobacterium*-mediated transformation. These differences occur not only among species, but also among cultivars/ecotypes of species (e.g., see 79). In addition to environmental or physiological factors, susceptibility to transformation has a genetic basis (9, 72, 79, 80). Therefore, specific host-encoded proteins contribute to the transformation process. During the past decade, scientists have utilized numerous approaches to identify these proteins.

A classic way of identifying genes and proteins involved in a biological process is through a forward genetic screening of mutant organisms to identify those with altered phenotypic characteristics. For plant biologists, the sequenced genome and extensive genomic resources make *Arabidopsis thaliana* the logical host of choice. Transformation of many *Arabidopsis* ecotypes (by either root or flower transformation protocols) is routine (21, 111), although the phenotypic and molecular results of using these target organs for transformation can differ (25, 49, 77). Because the natural site of *Agrobacterium*-mediated transformation is the root or the crown of the plant, forward genetic screening of *Arabidopsis* ecotypes (79) or mutants (80) has utilized root tissue rather than gametophytic tissue as the target for transformation. Although this approach is tedious, it has resulted in the identification of more than 125 loci affecting transformation (129). These screens have identified plants that are resistant to *Agrobacterium*-mediated transformation (*rat* mutants) (80, 129) or hypersusceptible to *Agrobacterium*-mediated transformation (*hat* mutants) (N Sardesai, H Chen, J Spantzel, B Yadav, SB Gelvin, in preparation). The mutagenized lines screened for altered

susceptibility include T-DNA disruption-tagged plants (1, 7, 39), T-DNA activation-tagged plants (121), and RNA interference (RNAi) lines directed against specific classes of *Arabidopsis* genes (**http://www.chromdb.org**; 22).

More recently, Anand et al. (6) used virus-induced gene silencing (VIGS) to identify genes important for *Agrobacterium*-mediated transformation in *Nicotiana benthamiana*, and other groups have used yeast as a plant surrogate to identify genes important for *Agrobacterium*-mediated transformation (14, 90, 112, 113, 114).

A number of virulence effector proteins are transferred from *Agrobacterium* to plant cells, and their interaction with plant proteins is likely important for the transformation process. Thus, in a second approach, numerous groups have conducted protein-protein two-hybrid interaction traps in yeast to identify, within a plant cDNA library, plant proteins that interact with specific transferred or surface-localized *Agrobacterium* Vir proteins (e.g., see 10, 11, 28, 54, 95, 101, 108). More recently, scientists have verified interaction of these plant proteins with *Agrobacterium* Vir proteins in planta using bimolecular fluorescence complementation (BiFC) (19, 65).

A third approach used to identify plant proteins that may be involved in *Agrobacterium*-mediated transformation involves transcriptional profiling. In these studies, scientists compared transcripts of plants subjected to *Agrobacterium* infection with those of noninfected plants or compared transcripts found within crown gall tumors with those from nontumorous tissues. These studies have been conducted in tobacco (116), *Ageratum* (30, 31), and *Arabidopsis* (27, 29, 56, 64). Many of these studies identified components of plant defense and hormone signaling pathways as either induced or repressed by *Agrobacterium* infection. In addition, many of the differentially regulated transcripts encode proteins previously identified as important for transformation. For example, genes encoding several histone proteins are induced within 24 h after *Agrobacterium* infection (116). Histones are important for transformation (6, 78, 102, 122, 123).

Finally, scientists have conducted reverse genetic experiments to determine whether particular genes are directly involved in the transformation process or are only accessory to or influenced by transformation. These studies targeted genes whose encoded proteins were identified in yeast two-hybrid screens, genes whose transcripts were differentially expressed after *Agrobacterium* transformation, or genes of interest whose importance in transformation was surmised. Typically, the expression of these genes was decreased by insertional mutagenesis, antisense RNA, RNAi, or VIGS or was increased by overexpression or by activation tagging. An effect on *Agrobacterium*-mediated transformation would indicate a direct role of these genes and their encoded proteins on transformation.

Throughout all these technological processes, a major argument to consider is whether the results of these experiments make biological sense. Although much still needs to be learned, the identification of the same genes and proteins putatively involved in transformation using several independent approaches leads us to conclude that many of these are truly involved in the transformation process directly. Below, I discuss what is known about these proteins from the perspective of how the transformation process progresses.

RNAi: RNA interference

VIGS: virus-induced gene silencing

cDNA: complementary DNA

BiFC: bimolecular fluorescence complementation

Stable transformation: *Agrobacterium*-mediated transformation of cells that results in integration of T-DNA into the host genome

Transient transformation: *Agrobacterium*-mediated transformation of cells that results in transgene transfer and expression without T-DNA integration

THE PATHWAY OF T-DNA AND VIRULENCE PROTEIN TRANSFER THROUGH THE PLANT

From the plant's perspective, there are eight major steps in the stable transformation process (six of these must occur for transient transformation). **Figure 1** presents these steps. Initially, *Agrobacterium* must attach to the plant cell surface, following which biofilm production frequently occurs. Second, T-DNA and Vir effector proteins are transported from the bacterium through the plant plasma membrane. Third and fourth, VirD2/T-strands

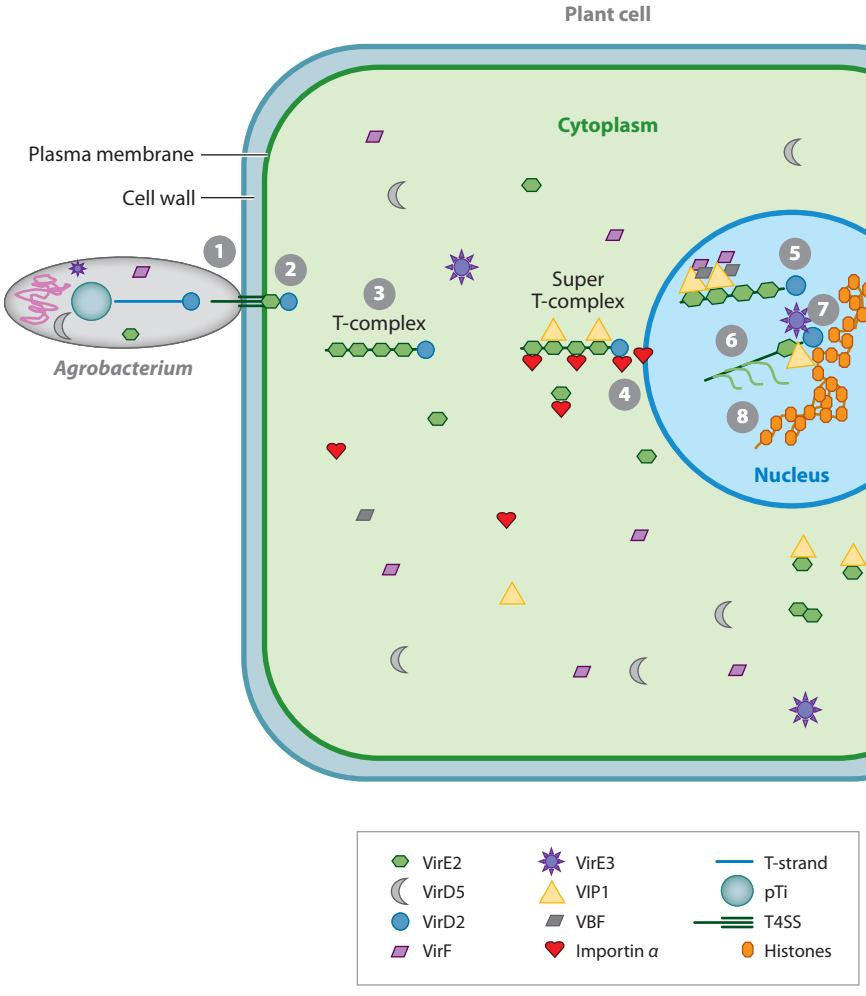

Figure 1

Schematic representation of the *Agrobacterium*-mediated plant transformation process. The names of several important virulence effector and plant proteins are noted. Circled numbers represent the following steps in transformation: 1. *Agrobacterium* attachment to the plant cell; 2. transfer of T-strands and virulence effector proteins through the plant plasma membrane into the plant cell; 3. T-complex and super-T-complex formation and subsequent cytoplasmic trafficking; 4. nuclear targeting; 5. targeting of the super-T-complex to chromatin; 6. removal of proteins from the super-T-complex prior to T-DNA integration; 7. T-DNA integration into the plant genome; 8. transgene expression. T4SS, type IV secretion system. The figure is not drawn to scale.

traffic through the plant cytoplasm and target the nucleus. Fifth and sixth, once inside the nucleus, the T-strand must target plant chromatin (not required for transient transformation), and the associated proteins must be stripped from the T-strand. Seventh, T-strands integrate into the host genome (this step is not required during transient transformation), and eighth, expression of T-DNA-encoded transgenes occurs. Throughout this process, VirD2/T-strands likely interact with other transferred *Agrobacterium* Vir proteins. The (still hypothetical) coating of T-strands with VirE2 protein, a single-strand DNA binding protein,

likely forms a T-complex within the plant cell. Further interaction of this T-complex with other *Agrobacterium* Vir proteins and with plant proteins results in the formation of super-T-complexes. Association and disassociation of T-strands with various *Agrobacterium* and plant proteins drives T-strand subcellular dynamics, ultimately taking it into the nucleus and to plant chromatin for integration.

Because the pathway for T-DNA transfer and trafficking through the plant is most likely a linear process, I discuss plant proteins involved in each of these steps sequentially. **Table 1** presents a summary of these steps and the known plant proteins involved.

Agrobacterium Attachment to Plant Cells

In nature, bacterial attachment to the plant cell is essential for T-DNA and Vir protein transfer. An exception to this situation can occur when *Agrobacterium* is microinjected into plant cells (38). All *Agrobacterium* mutants deficient in attachment are either avirulent or extremely attenuated in virulence (15, 33, 34, 55, 71, 84, 104). Early work described a proteinaceous substance on the plant surface (81, 82), a plant rhicadhesin binding protein (99), and a vitronectin-like protein (120) as important for bacterial attachment. However, these observations have not been confirmed, and a recent publication disputes the role of the vitronectin-like protein in bacterial attachment and *Agrobacterium*-mediated transformation (20). Several *Arabidopsis* ecotypes that are recalcitrant to transformation, such as Bl-1 and Petergof, bind *Agrobacterium* cells poorly (79). It is therefore likely that at least this first step of the transformation process is limiting with these plants. That this is true is demonstrated by the fact that RNAi-mediated decreased expression of a myb transcription factor gene (a negative regulator of transformation; see below) in ecotype Bl-1 increases both *Agrobacterium* attachment and transformation of the derived transgenic plants (N Sardesai & SB Gelvin, in preparation).

Several plant proteins have been implicated in *Agrobacterium* attachment to plant cells. Gaspar et al. (44) described an *Arabidopsis* arabinogalactan protein, AtAGP17, which is important for transformation. Arabinogalactan proteins are highly glycosylated proteins that are localized to plant cell walls or are secreted into the apoplast. They had previously been implicated in plant development and perhaps pathogenesis. An *AtAGP17* mutant, *rat1*, was identified in a screen for *Arabidopsis* T-DNA insertion mutants that had decreased susceptibility to *Agrobacterium*-mediated transformation. The *rat1* mutant is highly recalcitrant to transformation, and roots of these plants bind *Agrobacterium* cells poorly under all tested conditions (44, 80). Interestingly, AGP17 appears to be involved in host defense reactions and signaling. When wild-type *Arabidopsis* roots are incubated with *Agrobacterium*, a large decrease in expression of the plant defense genes *PR1* and *PR5* occurs within one hour. However, this marked decrease in defense gene expression did not occur when roots of *rat1* plants were infected by *Agrobacterium*. *Agrobacterium* infection of roots of wild-type *Arabidopsis* plants also resulted in decreased levels of salicylic acid (SA), a key plant defense signaling compound. The *rat1* mutant contains lower endogenous levels of SA, and infection by *Agrobacterium* did not lower SA levels to the extent that occurred in wild-type plants. Thus, AGP17 may play two roles in *Agrobacterium*-mediated transformation: AGP17 is important both for *Agrobacterium* attachment to roots and for the suppression of host defense responses.

It is interesting to note the relationship between *Agrobacterium* attachment to plant cells and plant defense signaling. Incubation of either *Agrobacterium* cells or plant roots with SA results in reduced binding of the bacteria to the plant and lower transformation efficiency (5, 124). Thus, *Agrobacterium* attachment may inhibit plant defense signaling. In addition to the role of AtAGP17 in attachment and defense signaling, an *Agrobacterium*-mediated alteration in plant defense gene expression has also been detected in tobacco (116), *Ageratum* (30, 31), and

T-complex: VirD2 protein covalently attached to the 5′ end of the T-strand, which is coated by VirE2 protein

Super-T-complex: hypothetical complex formed by association of host proteins and *Agrobacterium* virulence effector proteins with the T-complex

Table 1 Plant proteins and subcellular structures important for *Agrobacterium*-mediated transformation

Transformation process	Plant protein/structure involved	Reference
Bacterial attachment/biofilm formation	Arabinogalactan protein AtAGP17	44, 129
	Cellulose synthase-like CslA-09	128, 129
	Cellulose synthase-like CslB-05	N Sardesai & SB Gelvin, unpublished data
	Plant defense reaction proteins	5, 44, Veena & SB Gelvin, unpublished data
T-DNA and virulence protein transfer	Reticulon domain proteins BTI1 (AtRTNLB1), BTI2 (AtRTNLB2), and BTI3 (AtRTNLB4)	54, 129
	Rab8 GTPase	54, 129
Cytoplasmic trafficking	Microtubules[b]/kinesin	91, 129
	Actin and Myosin	129, P Rao, Y Yu, L-Y Lee, SB Gelvin, unpublished data
	Cyclophilin[a]	28
Nuclear targeting	Importin α	10, 11, 12, 65, 129
	Importin β/Transportin	129
	CAK2Ms kinase	10
	Protein phosphatase 2C (PP2C)	101
	VIP1	32, 50, 62, 66, 108
	Caspase[a]	16, 88
	GALLS interacting protein (GIP[a])	Y Wang, L-Y Lee, L Hodges, W Ream, SB Gelvin, unpublished data
Targeting T-DNA to chromatin	CAK2Ms[a]	10
	VIP1	61, 66, 69
Vir protein removal/T-DNA and protein stability	Ask/Skp proteins	95, 109, 125, 129
	Caspase	16, 88
	Histones	102
	pCsn5-1[a]	43
T-DNA integration	DNA ligase IV[a]	40, 112, 113, 114, 129, 130
	Ku70, Rad50, Mre11, Xrs2, Sir4[b]	112
	Ku80[a]	40, 42, 67
	VIP1	66
	VIP2	4
	Histones	6, 78, 80, 122, 123, 129
	Nucleosome assembly CAF-1	37
	Histone H3 chaperone SGA1	22, 129, G Tenea & SB Gelvin, unpublished data
	Histone deacetylases	22, 129
Transgene expression	Histones H2A, H3-11, and H4	102
Susceptibility to transformation	Myb transcription factor	N Sardesai, H Chen, J Spantzel, B Yadav, SB Gelvin, unpublished data

[a]Role in this transformation step is likely but not yet proven, or the literature indicates conflicting results.
[b]Shown in a *Xenopus* in vitro system only.
[c]Important for *Agrobacterium*-mediated transformation of yeast, but not yet shown in plants.

Arabidopsis (29) cells, and in *Arabidopsis* plants (64).

Plant defense responses likely play an important role in the early stages of transformation. Plants with debilitated defenses, especially through the inactivation of the SA signaling pathway, are hypersusceptible to *Agrobacterium*-mediated transformation (5, 64), whereas plants that have been chemically elicited to heighten defense responses (5) or that constitutively express defense genes (*cep* mutant) are resistant to transformation (Veena & SB Gelvin, unpublished data). We have recently identified an *Arabidopsis hat* mutant that overexpresses a UDP-glucosyltransferase gene (N Sardesai & SB Gelvin, in preparation). Expression of many defense genes is decreased in this mutant plant.

In addition to involvement of the plant cell wall structural protein AtAGP17 in *Agrobacterium*-mediated transformation, several cell wall carbohydrate biosynthetic enzymes also play a role in transformation. Mutation of the gene encoding the cellulose synthase-like protein CslA-09 (*rat4* mutant) reduces *Agrobacterium*-mediated transformation efficiency (128). *CslA-09* is highly expressed in the root elongation zone, the region that is preferentially susceptible to transformation (122). Disruption of the gene encoding CslB-05, however, increases transformation susceptibility of the plant almost twofold (N Sardesai & SB Gelvin, in preparation). Additional evidence for a role of plant proteins in *Agrobacterium* attachment comes from the *rat3* mutant (which has a reduced ability to bind bacterial cells under some conditions) (80) and the identification of a plant cell wall β-expansin mutant as a *rat* mutant (*ratT10*) (129). A VIGS approach to mutagenesis identified an α-expansin gene as important for *N. benthamiana* transformation (6). However, decreased expression of this gene did not inhibit *Agrobacterium* attachment to plant cells. Thus, the role of α-expansin in plant transformation likely occurs at some other early step of the transformation process (6).

T-DNA and Virulence Protein Transfer into Plant Cells

Although *Agrobacterium* attachment to plant cells is Ti-plasmid-independent and therefore mainly relies upon expression of bacterial chromosomal genes, productive attachment (i.e., binding of the bacteria that leads directly to T-DNA and virulence effector protein transfer) likely depends on host cell connections to the *Agrobacterium* T4SS. The T4SS includes a membrane transport channel and a T-pilus (41). The major pilin protein, VirB2, is a processed cyclic protein (36, 63). In addition, VirB5 is a minor component that localizes to the T-pilus tip (2), and some nopaline-type *Agrobacterium* strains produce a *trans*-zeatin synthase (Tzs) protein that associates with VirB5 on the bacterial surface (3). The function of the T-pilus remains unclear: It may merely have adhesin activity to bring the bacterial and plant membranes into close contact for T-DNA and Vir protein transfer (8), and/or it may serve as a conduit for T-DNA and Vir protein transfer.

To identify a potential plant receptor for the T-pilus, Hwang & Gelvin (54) conducted a yeast two-hybrid screen using processed (but not cyclized) VirB2 protein as bait and an *Arabidopsis* cDNA library. They identified three similar reticulon domain-like (RTNL) proteins, VirB2-interacting proteins BTI1, -2, and -3 (now termed AtRTNLB1, AtRTNLB2, and AtRTNLB4), and a Rab8 GTPase that interacted specifically with VirB2, with themselves, and with each other, but not with other Vir proteins. BTI-1/AtRTNLB1 localizes to the plant plasma membrane (54, 70), whereas BTI-2/AtRTNL2 and BTI-3/AtRTNL4 localize to the endoplasmic reticulum (83). The self- and hetero-interactions among these proteins suggest that they may form a complex at the plant plasma membrane to mediate T-DNA and Vir protein transfer into the plant. T-DNA knockout, antisense RNA, and RNAi inhibition of expression of the genes encoding these proteins resulted in reduced transformation efficiency, whereas overexpression of BTI-1/AtRTNL1 in

Table 2 Plant proteins whose overexpression increases *Agrobacterium*-mediated plant transformation

Protein	Likely role in transformation	Reference
BTI1 (AtRTNLB1)	T-DNA and virulence protein transfer	54
VIP1	Nuclear targeting	32, 109
Ku80	T-DNA integration	67
Histones H2A, H3-11, and H4	T-DNA stability, transgene expression	78, 102, 123, 127
SGA1 (ASF1)	T-DNA integration	G Tenea & SB Gelvin, unpublished data
UDP-glucosyltransferase	Defense response	N Sardesai & SB Gelvin, unpublished data
GALLS interacting protein (GIP)	Nuclear targeting[a]	Y Wang, L-Y Lee, L Hodges, W Ream, SB Gelvin, unpublished data

[a]Role in this transformation step is likely but not yet proven.

Arabidopsis increased transformation efficiency (**Table 2**). Interestingly, the level of BTI-1 protein increased soon after contact of plant cells with *Agrobacterium*. Pre-incubation of *Agrobacterium* with recombinant BTI-1 protein inhibited subsequent plant cell transformation, suggesting that BTI-1 interacted with and saturated a protein or structure on the bacterial surface important for transformation. However, BTI proteins have not been demonstrated to bind to T-pili. Reticulon domain proteins are ubiquitous in eukaryotes (85) and may serve as receptors for the T-pilus during the initial stages of *Agrobacterium*-mediated transformation of plants, fungi, and animal cells.

Little is known about the role of VirB5 in *Agrobacterium* attachment and T-DNA/Vir protein transfer to plant cells. However, the VirB5 ortholog CagL, which associates with the T4SS pilus of the human pathogen *Helicobacter pylori*, is a specialized adhesin that binds to the host integrin receptor and mediates the secretion of CagA effector protein into the host (59).

Cytoplasmic Trafficking and Nuclear Targeting

Once VirD2-T-strands and virulence effector proteins enter the plant cell, they must traffic through the cytoplasm and target the nucleus. All virulence effector proteins contain nuclear localization signal (NLS) sequences that are important for nuclear targeting. However, deletion of the C-terminal bipartite NLS of VirD2 has a relatively minimal effect on transformation (76, 96). This is likely because of built-in redundancy in the T-strand nuclear targeting process: According to a currently popular model, each T-strand is coated by hundreds of VirE2 molecules in the plant cell, and the two bipartite NLS sequences within VirE2 likely help target T-strands to the nucleus, even in the absence of VirD2 NLS sequences (45). In addition, T-strands (and their associated Vir proteins) can interact with plant proteins to form super-T-complexes. Many of these plant proteins facilitate nuclear targeting of karyophilic proteins and nucleic acid/protein complexes.

NLS: nuclear localization signal

The first identified class of plant proteins that mediate virulence effector protein (and, hence, T-strand) nuclear targeting were the importin α proteins. AtKAPα (now termed Impa-1) was identified in a yeast two-hybrid screen of an *Arabidopsis* cDNA library as a protein that interacts with the NLS peptide domain of VirD2 (11). Impa-1 was initially reported not to interact with VirE2 (11). However, several more recent reports indicate that Impa-1 (and all other investigated importin α family members; the *Arabidopsis* genome encodes nine importin α proteins) interact with both VirD2 (10, 12, 65) and with VirE2 (12, 65). Interaction of VirE2 with several importin α family members was demonstrated in vitro, in yeast, and (by BiFC) (19) in plant cells. In addition, VirE3, a protein that is exported from *Agrobacterium* to plant cells and localizes to the nucleus, interacts in yeast and in vitro with Impa-1 and Impa-4 (43).

In most examined eukaryotes (except yeast), importin α proteins are encoded by a multigene family. How important are each of these *IMPA* family members for *Agrobacterium*-mediated plant transformation? Bhattacharjee et al. (12) approached this problem by assaying *Arabidopsis* plant lines individually containing T-DNA insertions in various importin α genes or containing an RNAi construction that targeted a specific importin α gene. Despite the observation that all of the tested importin α proteins interact with both VirD2 and VirE2, mutation of *IMPA1*, *IMPA2*, or *IMPA3* had no effect upon *Agrobacterium*-mediated transformation. However, disruption of expression of *IMPA4*, either by T-DNA insertional mutagenesis or by RNAi-mediated inhibition, resulted in a large decrease in transformation frequency. Interestingly, transformation competence could be restored to the *impa4* mutant plant by introduction of any of six tested *IMPA* cDNAs under the control of a strong, constitutive promoter. Transcriptional profiling and promoter analyses indicated that the expression pattern of each *IMPA* gene differs (12). Thus, with regard to transformation, the various importin α proteins appeared functionally redundant. However, importin α genes under their native promoters, other than *Impa4*, could not rescue the *impa4* mutant plant because of differential promoter expression profiles.

In addition to importin α proteins, importin β-like transportins may also play a role in *Agrobacterium*-mediated transformation. A T-DNA insertion into the *Arabidopsis* importin β3 gene (*rat fl1* mutant) resulted in a rat phenotype (129).

Because of the initial perceived lack of interaction of VirE2 with AtKAPα (11), Tzfira et al. (108) conducted a yeast two-hybrid screen for proteins that would interact with VirE2 and, perhaps, mediate VirE2 nuclear targeting. They identified two VirE2 interacting proteins, VIP1 and VIP2. VIP1 interacted specifically with VirE2, but not with VirD2, in several different assay systems. VirE2 does not target nuclei of animal cells. However, coexpression of VIP1 with VirE2 resulted in nuclear localization of VirE2 in these cells (50). The authors therefore hypothesized that VIP1 also facilitates VirE2 nuclear import in plant cells. In accord with this model, nuclear import of VirE2 (but not VirD2) was inhibited in VIP1 antisense tobacco plants, as was *Agrobacterium*-mediated transient and stable transformation of these plants. Thus, VIP1 is important for transformation and functions most likely at the nuclear import step, although it may also play a role in T-DNA integration into the plant genome (see below). VIP1 is expressed at low levels in many plant species. Lacroix et al. (62) observed that the *Agrobacterium* effector protein VirE3 could transfer to plants and interact with VirE2. This result suggested that in plants containing limiting amounts of VIP1, VirE3 could facilitate VirE2 nuclear targeting in a manner similar to that of VIP1. Indeed, expression of VirE3 in VIP1 antisense tobacco plants restored both VirE2 nuclear import and transformation-susceptibility.

VIP1/2: VirE2 interacting proteins 1 and 2

The recent characterization of VirE2-importin α interaction (12, 65), as well as VIP1-importin α interaction (L-Y Lee & SB Gelvin, unpublished data) suggests that nuclear import of T-strands occurs as a super-T-complex composed of VirD2-T-strands, VirE2, VIP1, and importin α (and perhaps other) proteins.

The route by which super-T-complexes traverse the cytoplasm to target the nucleus is unknown. Phosphorylation of super-T-complex components is likely important for this targeting. VirD2 is a phosphoprotein that binds to and is phosphorylated by CAK2Ms, a cyclin-dependent kinase-activating kinase (10). VirD2 also interacts with a protein phosphatase 2C (PP2C), which can catalyze dephosphorylation of VirD2 (101). A β-glucuronidase (GUS)-VirD2 fusion protein localizes to the nucleus of tobacco BY-2 cells. However, overexpression of a tomato PP2C cDNA relocalized the fusion protein to the cytoplasm of these cells. Serine^{394} is located just upstream of the bipartite NLS of VirD2, and is the likely target of phosphorylation. Alteration of this serine residue to an alanine residue resulted

in decreased nuclear targeting of the GUS-VirD2 fusion protein, further implicating phosphorylation of serine[394] in VirD2 nuclear targeting. The importance of plant PP2Cs in *Agrobacterium*-mediated transformation is indicated by the observation that the *Arabidopsis abi1* mutant, which lacks a PP2C protein, is hypersusceptible to transformation (101).

Phosphorylation of VIP1 is also important for *Agrobacterium*-mediated transformation. Djamei et al. (32) demonstrated that VIP1 is phosphorylated on serine[79] by the mitogen-activated protein kinase MPK3. Both nuclear localization of VIP1 and *Agrobacterium*-mediated transformation are dependent upon VIP1 phosphorylation. These data suggest that VIP1 phosphorylation is important for super-T-complex nuclear targeting. Subcellular localization of VirE2 differs in diverse plant cells. Transgenic *Arabidopis*, expressing a VirE2-YFP fusion protein, displays cytoplasmic yellow fluorescence protein (YFP) fluorescence in root cells and in leaf mesophyll cells, but nuclear fluorescence in leaf trichome cells (12, 47). However, a correlation between VIP1 phosphorylation and VirE2 localization in these various cell types has not yet been established.

In addition to enzymes that can phosphorylate or dephosphorylate VirD2, Deng et al. (28) identified several cyclophilins that interact with VirD2 in yeast and in vitro. Bako et al. (10) also identified several cyclophilins in a yeast two-hybrid screen of *Arabidopsis* cDNAs using VirD2 and the bait protein. Although the role of cyclophilins in *Agrobacterium*-mediated transformation remains unknown, Deng et al. (28) suggested that they may be involved in mediating conformational changes in VirD2 necessary for subcellular trafficking. Indeed, incubation of plant cells with Cyclosporin A, a cyclophilin inhibitor, decreased transformation of *Arabidopsis* and tobacco cells. More recently, however, van Kregten et al. (115) demonstrated that the cyclophilin-interaction domain of VirD2 is not essential for virulence. Thus, the role of cyclophilins in *Agrobacterium*-mediated transformation remains unknown.

A role for plant caspases in *Agrobacterium*-mediated transformation has recently been described. Caspases are cysteine proteases that cleave proteins after specific aspartate residues. They are often associated with programmed cell death and hypersensitive responses. A tobacco caspase-like protease was recently characterized using VirD2 as the cleavage substrate (16). VirD2 is cleaved by this activity both in vitro and in vivo; two cleavage targets occur within the C-terminal region of nopaline-type VirD2 at amino acids 368–371 and 397–400. Cleavage at these sites would separate the T-strand-linked N-terminus of VirD2 from the C-terminal NLS sequences, thus possibly abrogating nuclear targeting of the T-complex. Mutation of the target aspartate residues to alanine in these two target sequences resulted in loss of VirD2 cleavage and increased transformation efficiency (88). The authors suggested that cleavage of VirD2 by plant caspase–like proteases may constitute a novel mechanism of plant defense against *Agrobacterium*-mediated transformation.

Although little definitive is known, it is possible that the plant cytoskeleton plays a role in T-complex cytoplasmic trafficking. Mutation of several actin genes expressed in roots (*act2* and *act7*), but not of the pollen-expressed *act12* gene, resulted in decreased root transformation (129; P Rao & SB Gelvin, unpublished data), suggesting the involvement of actin microfilaments in the transformation process. These results are in accord with recent data from our laboratory indicating that mutation of specific *Arabidopsis* myosin VIII and myosin XI genes severely decreases transformation susceptibility of roots of these mutant plants (Y Yu, V Dolja, SB Gelvin, unpublished data). However, other studies have implicated microtubules in *Agrobacterium*-mediated transformation. Mutation of an *Arabidopsis* kinesin gene results in a rat phenotype (129), and fluorescently-labeled single-strand DNA bound by VirE2 protein tracks along microtubules in a cell-free *Xenopus* egg extract (91).

Recently, Tenea et al. (102) demonstrated the importance of particular histone proteins

in protecting DNA introduced in plant cells either by *A. tumefaciens*- or electroporation-mediated transformation. Histone H2A-1 protein has been implicated in T-DNA integration into the plant genome (78; see below). Mutation of the *HTA1* gene (in the *rat5* mutant) decreases stable but not transient transformation of *Arabidopsis* roots (80, 129). However, overexpression of *HTA1* in transgenic *Arabidopsis* results in increased transient as well as in stable transformation (**Table 2**) (78, 102, 122). Because transient transformation does not require T-DNA integration, these results indicate that histone H2A-1 may play a role in transformation in addition to that of T-DNA integration. Indeed, introduction of the *HTA1* gene (which encodes a protein that localizes predominantly, but not exclusively, to plant nuclei) or several mutant *HTA1* derivatives (that encode H2A-1 proteins that localize to the plant cytoplasm) effected increased rates of transient transformation when cotransfected with a plant-active *gusA* gene (102). The H2A-1 protein and mutant derivatives also protected transfected DNA from nucleolytic degradation within the plant cell. Analysis of H2A-1 derivatives indicated that amino acids important for protein-DNA interaction were most important for this protection and for increased transgene expression. The observation that protection of introduced DNA could be mediated by histone H2A-1 derivatives that localize predominantly to the plant cytoplasm indicates that these proteins could function to protect transgene DNA in the cytoplasm. Interestingly, overexpression of all seven tested *Arabidopsis HTA* cDNAs, as well as the *HTR11* and *HFO* cDNAs (which encode histone H3–11 and histone H4), increased both *Agrobacterium*-mediated transformation and transgene expression. However, overexpression of none of the seven tested *HTB* cDNAs, nor of other tested *HTR* cDNAs, increased *Agrobacterium*-mediated stable transformation or transgene expression (**Table 2**; 102).

Additional plant proteins may also play a role in cytoplasmic trafficking of T-complexes through plant cells. Many *A. rhizogenes* strains lack *virE1* and *virE2*, genes that are essential for efficient transformation by *A. tumefaciens* strains (51). Rather, they contain a gene, *GALLS*, that produces two proteins, GALLS-FL (full-length) and GALLS-CT (a protein containing only the C-terminal region of GALLS, which initiates translation from an internal start codon within the *GALLS* gene) (52). GALLS can complement a *virE2 Agrobacterium* mutant for full virulence (51). GALLS-FL and GALLS-CT contain a T4SS sequence required for export into plant cells. However, they otherwise do not resemble VirE2 protein (53). The differences between GALLS proteins and VirE2 suggest that they mediate transformation by different mechanisms, and thus may interact with different plant proteins. A yeast two-hybrid screen of *Arabidopsis* cDNAs, using GALLS-FL protein as bait, identified a small protein, GIP (GALLS interacting protein) (Y Wang, L-Y Lee, LD Hodges, W Ream, SB Gelvin, in preparation). GIP belongs to a 10-member *Arabidopsis* gene family whose only other characterized member, *LSH1*, is important for light regulation of seedling development (126). GIP interacts with GALLS-FL in the nucleus and with GALLS-CT in the cytoplasm. Interestingly, overexpression of GIP in transgenic *Arabidopsis* enhances both GALLS- and VirE2-mediated *Agrobacterium*-mediated transformation (**Table 2**). BiFC experiments indicate that GIP can interact with VirE2 as well as with GALLS proteins (L-Y Lee & SB Gelvin, unpublished data). The precise transformation step at which GIP functions is not yet known.

GIP: GALLS interacting protein

Targeting T-Strands to Plant Chromatin

Once within the nucleus, T-strands must target plant chromatin prior to integration (a step not required for transient transformation). Several plant proteins, in affiliation with virulence effector proteins, may facilitate this step. As described above, Bako et al. (10) identified a kinase, CAK2Ms, that phosphorylates VirD2. CAK2Ms also phosphorylates the largest

subunit of RNA polymerase II. Phosphorylation of this subunit recruits the TATA box-binding protein, and transcription can thus initiate. Interestingly, VirD2 can also associate with the TATA box-binding protein (10). The authors suggested that the association of VirD2 with the TATA box-binding protein may guide T-strands to transcriptionally active regions of chromatin for integration. Although surveys of several T-DNA tagged libraries in *Arabidopsis* and rice suggested that T-DNA preferentially integrates into promoter regions of genes and into transcriptionally active chromatin (e.g., 13, 68, 93, 100), Kim et al. (56) demonstrated that this seeming integration site preference was an artifact resulting from selection bias when regenerating transgenic plants. Rather, Kim et al. (56) concluded that, without selection for recovery of transgenic plants, T-strands integrate randomly into the *Arabidopsis* genome without regard to DNA sequence, transcriptional activity, or DNA methylation status. Thus, targeting of potential T-DNA integration sites may depend more upon general chromatin proteins (such as histones) than upon proteins specific for transcription.

Indeed, the potential role of histones in targeting T-DNA to chromatin integration sites was suggested by several studies in which VIP1 protein was shown to interact with various histones (66, 69). The C-terminal region of VIP1 is required for this interaction (66). This region of VIP1 is also important for T-DNA integration (see below). Not only can VIP1 interact with histones, it also directs in vitro interaction of T-DNA complexes consisting of single-strand DNA, VIP1, and VirE2 with nucleosome monomers (61). Thus, VIP1 may act as a molecular bridge to guide T-strands to plant chromatin for subsequent integration.

Stripping Proteins from T-Strands

Prior to or during the process of T-DNA integration, both *Agrobacterium* virulence effector proteins and associated plant proteins must be removed from T-strands. Schrammeijer et al. (95) first proposed a role for the plant proteosome degradation pathway in this process. Using a yeast two-hybrid system, they identified three Skp-1 related proteins, ASK1, ASK2, and ASK10, that interact with the virulence effector protein VirF. VirF is important for efficient transformation by many *Agrobacterium* strains. Mutation of *virF* in *Agrobacterium* can be complemented by expression of *virF* in plants, showing that VirF protein functions within the plant cell (89). VirF is a F-box protein that is likely incorporated into SCF complexes important for tagging proteins with ubiquitin and targeting them for proteolysis by the 26S proteosome. Subsequent research has indicated that VirF likely plays an important role in removal of proteins from T-complexes. Tzfira et al. (110) showed that VirF is important for targeted proteolysis of VirE2 and VIP1 in both yeast and plant cells. Inhibition of 26S proteosome activity resulted in decreased transient expression of an introduced *gusA* gene. VirF also associates with T-strand/VIP1/VirE2/nucleosome complexes in vitro, suggesting that VirF is targeted to nucleosomes in order to facilitate proteolysis of T-complex-associated proteins prior to T-DNA integration into the plant genome (61).

Some *Agrobacterium* strains lack *virF* and are weakly virulent on some plant species such as tomato (74). The high level of virulence of these strains on other species, however, suggests that these species may encode a functional equivalent of VirF. Zaltsman and coworkers recently described an *Arabidopsis* gene, *VBF*, which serves this function (125). VBF (VIP1-binding F-box protein) encodes a F-box protein that interacts with VIP1 and forms a ternary complex with VIP1 and VirE2 in plant nuclei. Complex formation resulted in degradation of both VIP1 and VirE2 via the SCF pathway. Interestingly, antisense inhibition of VBF expression in transgenic *Arabidopsis* resulted in hyperaccumulation of VIP1 but lower transformation susceptibility of these plants, suggesting that degradation of VIP1 is important for transformation to succeed. The authors also demonstrated the functional

equivalency of VBF and VirF by expressing a VBF derivative containing a T4SS export signal in a *virF* mutant *Agrobacterium* strain. Expression of this VBF derivative restored high virulence to the *virF* mutant strain when assayed on tomato.

VirE3 protein interacts with VirE2 and may substitute for VIP1 in plants (62). In yeast and in vitro, VirE3 protein can interact with *Arabidopsis* pCsn5-1 protein (43). pCsn5-1 forms part of the COP9 signalosome and may regulate protein stability. It is not known whether this interaction results in increased or decreased VirE3 stability in plant cells or whether VirE3 is found as part of super-T-complexes.

T-DNA Integration into the Plant Genome

The mechanism of T-DNA integration into the plant genome remains controversial. Two major models have been proposed (107). The first, the strand-invasion model, posits that VirD2-T-strands search for regions of microhomology between T-DNA and plant DNA sequences. Using this homology, T-strands locally invade and melt out regions within target site host DNA. The presence of VirD2 on the 5′ end of the T-strands causes a nick to occur in one strand of plant DNA, into which the T-strand is ligated. During replication, the complementary strand of T-DNA is synthesized, resulting in incorporation of a double-strand copy of the T-strand into the plant genome (105). The second model, double-strand break repair and integration, hypothesizes that single-strand T-strands are replicated in the plant nucleus to a double-strand form that subsequently integrates into double-strand breaks in the host genome. This model requires a nonhomologous end-joining (NHEJ) process. The double-strand break repair model better explains the presence of inverted repeat copies of T-DNA that frequently are found in transgenic plants, and the fact that different T-DNAs introduced into the same plant cell by different *Agrobacterium* strains frequently link together upon integration (24, 26, 60). In addition, double-strand breaks deliberately introduced into plant DNA provide preferential T-DNA integration targets (17, 92, 106).

NHEJ utilizes a number of proteins to repair the double-strand break, including Ku70, Ku80, XRCC4, and DNA ligase IV. However, the role of these proteins and of *Agrobacterium* virulence effector proteins in T-DNA integration remains elusive. VirD2 is known to possess a DNA ligase activity (87). However, in vitro ligation of a VirD2-oligonucleotide model substrate to an acceptor DNA substrate required a plant ligase activity and not that of VirD2 (130). The nature of this plant ligase activity remains unknown. For example, *Arabidopsis thaliana* encodes one DNA ligase IV gene. Mutation of this gene, however, had either little (40) or no effect upon *Agrobacterium*-mediated root or flower-dip transformation (113, 114) of *Arabidopsis* plants, although it is required for T-DNA integration in yeast (112). Other NHEJ proteins, including Ku70, Rad50, Mre11, Xrs2, and Sir4, are also required for T-DNA integration in yeast (112).

The role of Ku80 in T-DNA integration also remains controversial. Using *ku80* mutant plants, Gallego et al. (42) saw a twofold increase in the frequency of *Arabidopsis* flower-dip transformation, whereas Friesner & Britt (40) saw a two- to threefold decrease in flower-dip transformation using a different *ku80* mutant. However, both of these studies used a flower-dip transformation protocol to quantify transformation frequency. It is known that female gametophytic transformation, which occurs during the flower-dip protocol, does not reflect the same process observed using a root segment somatic cell transformation protocol (77). Using a root transformation assay, Li et al. (67) demonstrated that an *Arabidopsis ku80* mutant was severely reduced in its ability to support stable, but not transient, *Agrobacterium*-mediated transformation. Complementation of the mutant with a wild-type *Ku80* gene restored transformation susceptibility to the mutant plants, whereas overexpression of the *Ku80* gene in transgenic *Arabidopsis* increased stable but not transient

transformation (**Table 2**). The finding that transient transformation of the *ku80* mutant and overexpressing lines was similar to that of wild-type plants, but that stable transformation was altered, suggested that Ku80 functions at the step of T-DNA integration but not at prior steps in the transformation process. Additional experiments indicated that T-DNA could be immunoprecipitated from transgenic *Arabidopsis* cells overexpressing His-tagged Ku80 protein and that these plants also link two T-DNA molecules together. Finally, the authors demonstrated that overexpression of Ku80 increased the extent of T-DNA integration into the genome. Taken together, the results of Li et al. (67) suggest that Ku80 plays a direct role in T-DNA integration into the plant genome.

VIP1 and VIP2 proteins also play a role in T-DNA integration. The role of VIP1 in integration was originally masked by its importance in T-DNA nuclear targeting (108). Thus, if the T-complex could not reach the nucleus in *vip1*$^-$ mutant plants, it would be difficult to determine whether important T-complex protein constituents also facilitated T-DNA integration. Li et al. (66) characterized an *Arabidopsis* T-DNA insertion into the *VIP1* gene. This insertion caused the truncation of VIP1 protein. However, the N-terminal fragment of VIP1 could still accumulate in cells. The authors determined that this N-terminal fragment could still interact with VirE2 protein and direct T-strands to plant nuclei, permitting transient transformation to occur. However, truncated VIP1 could not multimerize, nor could it interact with histones. The result of this mutation of VIP1 thus was a deficiency in stable transformation most likely caused by decreased T-DNA integration into the plant genome.

Characterization of an *Arabidopsis vip2* mutant, as well as VIGS-mediated suppression of VIP2 expression in *N. benthamiana*, indicated that VIP2 is also involved in T-DNA integration (4). VIP2 interacts with VirE2. A *vip2* mutant was fully susceptible to transient transformation, whereas *vip2* mutant and VIGS-silenced plants were attenuated in their susceptibility to stable transformation. Molecular and genetic analyses indicated that T-DNA integration was blocked in *vip2* mutant plants. VIP2 is likely a transcriptional regulator. Microarray analysis indicated that *vip2* mutant plants have altered transcriptional profiles, including lower levels of histone transcripts. Given the importance of many histone genes in T-DNA integration (see below), these data suggest that VIP2 is important for regulating the expression of genes involved in *Agrobacterium*-mediated transformation.

Histone proteins play a major role in T-DNA integration into the plant genome. The *Arabidopsis rat5* mutant, which contains an insertion into the 3′ untranslated region of the histone H2A-1 gene *HTA1*, is susceptible to transient transformation but highly resistant to stable transformation (78, 80, 129). This recalcitrance to stable transformation results from a decrease in T-DNA integration into the plant genome (78). *HTA1* encodes a replacement histone that is expressed at low levels in all cells examined. Interestingly, *HTA1* is most highly expressed in *Arabidopsis* cells of the root elongation zone, the region of the root that is most susceptible to transformation (122). *HTA1* is one of a 13-member histone H2A (*HTA*) gene family. The observation that the *hta1* (*rat5*) mutant is resistant to *Agrobacterium*-mediated transformation suggests lack of functional redundancy among the *HTA* genes. Interestingly, the *rat5* mutant can be complemented to transformation-susceptibility by constitutive overexpression of many of the other *HTA* cDNAs, indicating that histone proteins, as opposed to histone genes, may be functionally redundant with regard to transformation (123). The promoters of the various *Arabidopsis HTA* genes show partially overlapping but distinct patterns of expression, suggesting that the lack of functional redundancy among the *HTA* genes results from differential patterns of histone H2A gene expression. Indeed, only the *HTA1* promoter could respond to wounding or *Agrobacterium* infection of root tissue (123). Thus, the *HTA1* gene shows special properties that make it important for *Agrobacterium*-mediated transformation.

The importance of histone genes in *Agrobacterium*-mediated transformation is also reflected by the number of T-DNA insertions in *Arabidopsis* histone genes that result in the rat phenotype. These include mutation of various histone H2A, H2B, H3, and H4 genes (129). Although many of these mutations have not been fully characterized, the rat phenotype caused by a T-DNA insertion between the closely spaced *HTR4* and *HTR5* genes can be complemented by a genomic fragment containing both of these genes (Y Zhu & SB Gelvin, unpublished data). In addition, VIGS analysis indicated that *HTA* and *HTR* genes are important for transformation of *N. benthamiana*. In particular, suppression of *HTR* gene expression decreased T-DNA integration into the plant genome (6).

In addition to the role of histones in T-DNA integration, histone chaperones also are important for *Agrobacterium*-mediated transformation. The chromatin assembly factor complex CAF-1, made up of three subunits, is important for nucleosome assembly. Mutation of the genes encoding two of the subunits, *fas1* and *fas2*, resulted in increased susceptibility of roots of these plants to *Agrobacterium*-mediated transformation (37). The authors speculated that a decreased rate of loading nucleosomes onto DNA during the process of chromatin assembly may transiently leave the plant DNA naked and therefore more accessible to T-DNA integration. Conversely, Crane & Gelvin (22) described three *Arabidopsis* lines in which expression of the histone H3 chaperone gene *SGA1* had been decreased by RNAi inhibition. Although these plants were normal in growth and development, they were highly resistant to *Agrobacterium*-mediated transformation. By examining the amount of T-DNA integrated into the genome of these and wild-type plants, the authors demonstrated that the block to transformation of these plants was at the T-DNA integration step. Overexpression of *SGA1* enhances *Agrobacterium*-mediated transformation by increasing the amount of integrated T-DNA (**Table 2**) (G Tenea & SB Gelvin, unpublished data).

Numerous other genes that contribute to chromatin structure and function also affect *Agrobacterium*-mediated transformation. These include histone deacetylases and acetyltransferases, nucleosome assembly factors, SET domain proteins, and DNA methyltransferases (22, 129). Although the precise roles of most of these proteins in the transformation process have not yet been established, the histone deacetylases HDT1 and HDT2 are important for T-DNA integration into the plant genome (22).

Transgene Expression

Overexpression of the histone H2A-1 gene *HTA1* results in increased *Agrobacterium*-mediated transformation (**Table 2**) (78, 127). Because loss of *HTA1* expression in the *rat5* mutant resulted in decreased T-DNA integration and thus transformation, a logical argument would be that increased transformation caused by *HTA1* overexpression results from increased T-DNA integration. However, overexpression of *HTA1* also increases the frequency of transient *Agrobacterium*-mediated transformation, a process that does not require T-DNA integration (76, 79, 102). Therefore, it is not likely that this hypersusceptibility to transformation (HAT phenotype) results from increased T-DNA integration. Recently, Tenea et al. (102) demonstrated that individual overexpression of seven different *HTA* genes, and the histone H3 (*HTR*) and H4 (*HFO*) genes *HTR11* and *HFO3*, increased transformation of *Arabidopsis* root segments (**Table 2**). However, individual overexpression of seven different H2B (*HTB*) genes and three additional *HTR* genes did not result in a HAT phenotype. Interestingly, overexpression of only the *HTA*, *HFO*, and *HTR11* genes increased transgene expression in a transient transformation system (102). In these experiments, a *gusA* reporter gene was introduced with each of the tested histone genes into tobacco BY-2 protoplasts, and GUS expression was assayed approximately 18 h later. This increase in transgene expression resulted

from increased transgene stability: When the *HTA*, *HFO*, and *HTA11* genes were cointroduced with the *gusA* DNA, more *gusA* DNA was detected within the protoplasts 24 h after transfection. Thus, particular histones were able to increase transgene expression (and therefore appeared to increase the frequency of transformation) because they could protect incoming transgene DNA from nucleolytic degradation. Overexpression of *HTA1* could not increase expression of previously integrated transgenes. Mutational analysis indicated that amino acid residues within histone H2A-1 that are important for this protection map to regions of the protein known to be important for histone-DNA interaction (102). This study demonstrated the importance of particular histones for incoming transgene stability and consequently, transgene expression.

Gelvin & Kim (48) recently summarized the role of chromatin in *Agrobacterium*-mediated transformation. It is likely that many of the genes described in Gelvin & Kim (48) will contribute to transgene expression as well as to T-DNA integration.

INTERACTION OF *AGROBACTERIUM* ONCOPROTEINS WITH PLANT PROTEINS

T-DNA of *A. tumefaciens* and *A. rhizogenes* encode a number of oncoproteins. These proteins are not important for T-DNA transfer or subcellular trafficking in the plant cell, but they are important for crown gall or hairy root tumorigenesis. Some of these proteins, such as gene *6b* of *A. tumefaciens* and *rolB* of *A. rhizogenes*, localize to plant nuclei. Interaction of these bacterial oncoproteins with plant proteins is important for both nuclear (and subnuclear) localization and for oncoprotein function (57, 58, 75, 103). Gene 6b protein interacts with the plant transcription factor NtSIP1 (57), the nucleolar-localized protein NtSIP2, and histone H3 (58, 103) and, as such, acts as a histone H3 chaperone. RolB protein interacts with the tobacco protein Nt14-3-3-ωII; this interaction is important for RolB nuclear localization (75).

GLOBAL REGULATION OF *AGROBACTERIUM*-MEDIATED TRANSFORMATION

Many plant species are highly susceptible to *Agrobacterium*-mediated transformation, whereas others are highly resistant. While assaying a T-DNA insertion library of *Arabidopsis* mutants, our laboratory recently identified a myb transcription factor gene that, when mutated, results in an approximately tenfold increase in transformation (N Sardesai, H Chen, J Spantzel, SB Gelvin, in preparation). RNAi-mediated inhibition of expression of the myb gene in the transformation-recalcitrant ecotype Bl-1 resulted in greatly increased transformation frequency of root segments of the derived transgenic lines. The transcriptome of myb mutant plants was altered in expression of approximately only 100 genes. Interestingly, many of these genes are involved in host defense against pathogens. Thus, this myb transcription factor may reduce susceptibility to *Agrobacterium*-mediated transformation by activating host defense responses to *Agrobacterium*.

FUTURE PROSPECTS

Agrobacterium-mediated transformation is complex and requires the activity of numerous plant proteins. In this review, I described many of these proteins and their known or presumed activities in the transformation process. However, it is clear that many plant genes important for transformation have yet to be identified. Only a small percentage of the already-identified *rat* mutants have been characterized (80, 129), and many techniques such as VIGS (6) have not yet been fully exploited.

In addition to those described in this review, it is likely that emerging technologies will add to our understanding of the transformation process. These technologies include proteomic analyses and metabolic profiling. This latter approach has already contributed to our

knowledge of crown gall tumorigenesis (27, 35). In addition, continued exploration of the role of plant defense responses to *Agrobacterium* (64) will indicate how host plants respond to infection by this biotrophic pathogen.

Finally, several novel technologies are currently being developed that will be useful for identifying plant proteins important for *Agrobacterium*-mediated transformation. These include peptide aptamer mutagenesis (a technique by which the expression of small peptides phenocopies mutations) to identify specific domains of plant proteins important for the transformation process and the development of a plant two-hybrid system to investigate protein-protein interactions directly in plants.

SUMMARY POINTS

1. *Agrobacterium*-mediated plant genetic transformation is a complex process requiring the concerted function of both bacterial virulence effector proteins and plant proteins.
2. During the past decade, scientists have utilized a number of techniques to identify and characterize plant genes and proteins important for *Agrobacterium*-mediated transformation. These methodologies include forward genetic screens to identify plant mutants that are resistant or hypersusceptible to transformation, yeast two-hybrid analyses to identify cDNAs encoding plant proteins that interact with transferred *Agrobacterium* virulence effector proteins, microarray and bioinformatics approaches to identify plant genes that are rapidly up- or down-regulated after *Agrobacterium* infection, and reverse genetic approaches to investigate the importance of specific plant genes in the transformation process.
3. From the plant's perspective, stable genetic transformation is a linear process involving (in order) *Agrobacterium* attachment, T-DNA and virulence effector protein transfer to the plant, cytoplasmic trafficking and nuclear targeting of T-DNA and associated proteins, targeting chromatin within the nucleus, stripping proteins from T-strands, T-DNA integration, and expression of T-DNA-encoded transgenes. Transient transformation does not require chromatin targeting or T-DNA integration into the host genome.
4. Sub-cellular trafficking of T-strands likely involves formation of T-complexes composed of T-strands capped with VirD2 and coated by VirE2 proteins. T-complexes likely form super-T-complexes by association with plant proteins that facilitate the steps outlined above.
5. Different plant species, or cultivars/ecotypes of a given species, frequently display various degrees of susceptibility to *Agrobacterium*-mediated transformation. Decreased expression or overexpression of more than 100 plant genes can alter susceptibility.

FUTURE ISSUES

1. Although several host proteins important for *Agrobacterium*-mediated plant transformation have been identified, many still await identification and characterization. Do monocots, especially cereal species, utilize for transformation the same plant proteins as those identified in dicot species?

2. To date, scientists have manipulated the expression of a few plant genes to increase the frequency of *Agrobacterium*-mediated transformation. How can plant genes be manipulated to improve the quality of transformation events (low integrated T-DNA copy number, targeting of T-DNA to specific chromatin regions or DNA sequences, predictable and stable transgene expression)?
3. Little is currently known about the mechanism of T-DNA integration into the plant genome. Which plant proteins participate in this process, and do their functions for integration mimic or extend their normal roles in processes such as DNA repair and recombination, DNA replication, and transcription?
4. *A. tumefaciens* VirE2 and *A. rhizogenes* GALLS proteins do not resemble each other, yet GALLS can substitute for VirE2 in *Agrobacterium*-mediated transformation. Do these two proteins mediate different routes or mechanisms of T-strand trafficking within plant cells? Which plant proteins are important for VirE2- and GALLS-mediated transformation?
5. How have *Agrobacterium* species and plants coevolved to permit transformation to occur under some circumstances but to limit transformation under other circumstances? Which *Agrobacterium* and plant proteins trigger or suppress plant defense responses?

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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